Low affinity of *Trypanosoma brucei* transferrin receptor to apotransferrin at pH 5 explains the fate of the ligand during endocytosis

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Abstract Uptake of host transferrin (Tf) in *Trypanosoma brucei* is mediated by a heterodimeric, glycosyl-phosphatidylinositol-anchored receptor. After endocytosis, Tf is delivered to lysosomes where it is proteolytically degraded. So far, the sequence of events leading to ligand dissociation and degradation is undefined. We now show by Triton X-114 phase separation that iron-free Tf (apo-Tf) dissociates from the receptor at pH 5.0. The low affinity of apo-Tf for its receptor at pH 5.0 is confirmed by an apparent dissociation constant of 1.1 μM. The implications of this result on the mechanism of intracellular processing of Tf in trypanosomes are discussed.

Key words: Transferrin receptor; Transferrin; *Trypanosoma brucei*

1. Introduction

The transferrin receptor (TfR) of *Trypanosoma brucei* differs in primary structure, subunit organisation and mode of membrane anchorage from the mammalian TfR. The trypanosome TfR is a heterodimeric complex of very low abundance (about 3000 molecules/cell) encoded by two expression site-associated genes (ESAGs), ESAG6 and ESAG7 [1–5]. The ESAG6 product (pESAG6) is a heterogeneously glycosylated protein of 50–60 kDa modified by a glycosyl-phosphatidylinositol membrane anchor, while the ESAG7 product (pESAG7) is a 42 kDa glycoprotein carrying an unmodified COOH-terminus [1]. Binding of one molecule of transferrin (Tf) [2] requires association of both pESAG6 and pESAG7 as shown by coexpression in heterologous systems [3–5]. Despite the profound difference in receptor structure, the apparent dissociation constant (K_d) for iron-loaded Tf (holo-Tf) is of the same order of magnitude for both the trypanosome TfR (3.6–108 nM [2]) and the mammalian TfR (2–110 nM [6,7]). The fate of Tf differs, however, in mammalian cells and trypanosomes. In mammalian cells, the Tf-TfR complex is transported to endosomes where Tf releases its iron. The iron-free Tf (apo-Tf) remains bound to its receptor and is recycled back to the cell surface where it dissociates from the receptor [6,8]. In contrast, Tf is delivered in trypanosomes to lysosomes where it is proteolytically degraded [2,9]. While the degradation products are released from the cells, iron remains cell associated [2]. As the sequence of events leading to ligand dissociation and degradation in *T. brucei* is undefined, we have studied the affinity of Tf for the trypanosome TfR at different pH values by Triton X-114 phase separation [10] and by determination of K_d values using membranes of trypanosomes. The results allow us to explain the intracellular processing of Tf in trypanosomes as compared to that of mammalian cells.

2. Materials and methods

2.1. Reagents

Bovine holo-transferrin (holo-Tf), *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (lauryl sulfobetaine) and *p*-chloromercuribenzenesulfonic acid (PCMBS) were purchased from Sigma, Deisenhofen, Germany; Triton X-114 from Serva, Heidelberg, Germany; and sodium boro[^{3}H]hydride (31 Ci/mmol) from Amersham, Braunschweig, Germany.

2.2. Trypanosomes

Variant clone MITat 1.4 of *T. brucei* strain 427 (117a [11]) was grown in NMRI mice and purified from blood by DEAE-cellulose chromatography [12].

2.3. Immobilisation and radioactive labeling of Tf

Holo-Tf was labeled with ^{3}H by reductive methylation using sodium boro[^{3}H]hydride as described previously [2]. Tf-Sepharose was prepared by coupling holo-Tf to CNBr-activated Sepharose 4B (Pharmacia, Freiburg, Germany) as described by the manufacturer.

2.4. Purification of trypanosome TfR and Triton X-114 phase separation

Trypanosomes (10^9/ml) were lysed by sonication in lysis buffer (50 mM HEPES, pH 7.0, 2.5 mM EDTA, 2 mM EGTA, 200 μM TLCK, 400 μM PMSF, 10 μM leupeptin, 2 μM E-64, 1 μM pepstatin A) containing 10 mM PCMBS and 2% lauryl sulfobetaine in order to inhibit cleavage of glycosyl-phosphatidylinositol membrane anchors by the endogenous phospholipase C [1]. After centrifugation at 114000×*g* for 1 h, TfR was precipitated from the supernatant with holo-Tf-Sepharose (25 μl of beads/10^9 cell equivalents) at 4°C by end-over-end rotation for at least 12 h. The beads were washed five times with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2) containing 0.2% Triton X-100 and the TfR was eluted from the Tf-Sepharose by shaking for 15 min at room temperature with 50 mM glycine, 150 mM NaCl, 0.2% Triton X-100, pH 2.7. The acidic eluate was immediately neutralised with 1 M Tris-HCl, pH 8.0 and incubated on ice for 1 h with excess holo-^{3}H-Tf in the presence of a 0.1 volume of 12% Triton X-114 (precondensed according to [10]). Temperature-induced phase separation [10] was performed by heating to 37°C for 30 min. After centrifugation for 5 min at 14000×*g*, the aqueous phase was discarded and the detergent-rich phase was washed once with prewarmed PBS. The detergent-rich phase was then adjusted to the original volume with (i) PBS, pH 7.2, (ii) 100 mM citrate, pH 5.0 or (iii) PBS, pH 5.0, and incubated on ice for 1 h. After heating to 37°C, the aqueous phase was removed and the detergent-rich phase was again adjusted to the original volume with the corresponding buffer. After a 1 h incubation, phase separation was induced again. Background values were determined by performing all Triton X-114 phase separation steps described above with radiolabeled Tf in the absence of TfR. Finally, the combined aqueous and detergent-rich phases were analysed by immunoblotting or processed for liquid scintillation counting.

2.5. Binding assays

Binding studies were performed with membranes from trypano-

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somes prepared by lysis of cells in lysis buffer on ice in the presence of 10 mM PCMBS with occasional shaking until no intact cells could be observed microscopically [2]. After centrifugation for 10 min at $14000 \times g$, the membrane pellet was washed twice with ice-cold 50 mM glycine, 150 mM NaCl, pH 3.5 and resuspended in 100 mM citrate, pH 5.0 or PBS, pH 5.0 by Dounce homogenisation. Membranes (1.5×10^8–10^9 cell equivalents/ml) were incubated with varying amounts of holo-^{3}H-Tf in the presence of 1 mg/ml fish gelatin by end-over-end rotation at room temperature for 1 h. After centrifugation for 5 min at $14000 \times g$, the membrane pellets were washed once with 1 ml ice-cold PBS. Then, the pellets were dissolved in 0.2 ml 2% SDS by heating to 100°C and the tubes were rinsed once with 0.1 ml 2% SDS. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled holo-Tf. The bound radioactivity was determined by liquid scintillation counting in 4 ml Rotiszint 22 (Roth, Karlsruhe, Germany).

3. Results

In order to study the dissociation of Tf from the trypanosome TfR at different pH values we subjected the ternary complex to Triton X-114 phase separation [10] and analysed the distribution of the amphiphilic form of the receptor and its ligand in the resulting detergent-rich and aqueous phases. The receptor was first purified under conditions where the endogenous phospholipase C was inhibited [1] and then saturated with its ligand by incubation with excess radiolabeled holo-Tf. After removing unbound Tf by Triton X-114 phase separation, the detergent-rich phase was mixed with (i) PBS, pH 7.2, (ii) citrate buffer, pH 5.0 or (iii) PBS, pH 5.0. Subsequently, a phase separation was induced again and the obtained phases were analysed by counting radioactivity or immunoblotting. At pH 7.2 in PBS, the ternary complex partitioned into the detergent-rich phase as most of the Tf (89%; Fig. 1a) and the amphiphilic TfR (Fig. 1b, compare lanes 1 and 2) were found in this phase. In citrate buffer at pH 5.0, nearly all Tf (96%) was associated with the aqueous phase (Fig. 1a) while again the receptor partitioned into the detergent-rich phase (Fig. 1b, compare lanes 3 and 4). As incubation of holo-Tf at pH 5.0 in the presence of citrate leads to liberation of iron [13,14], the above result suggests that Tf in its iron-free form (apo-Tf) dissociates from the receptor at this pH. In contrast, when the incubation was performed in PBS at pH 5.0, half of the Tf (51%) was associated with the detergent-rich and half (49%) with the aqueous phase (Fig. 1a) indicating that in the absence of citrate Tf releases less of its bound iron.

The above results suggest that apo-Tf has a low and holo-Tf a high affinity for its receptor at pH 5.0. Binding experiments of radiolabeled Tf to TfR in membranes from trypanosomes at pH 5.0 in citrate buffer or PBS showed this directly. Scatchard analysis of the binding data revealed 2400 binding sites for apo-Tf per cell and an apparent K_d value of 1.1 μM (Fig. 2a; citrate, pH 5.0) while 2300 binding sites and a K_d value of 12 nM were found for holo-Tf (Fig. 2b; PBS, pH 5.0). These K_d values are about 300- and 3-fold higher than recently reported for holo-Tf at pH 7.2, respectively (3.6 nM [2]). It should be noted that the ionic strength of the citrate buffer and PBS at pH 5.0 is similar (220 mM and 150 mM, respectively). The number of Tf binding sites present in the membrane preparations at pH 5.0 was identical to the number previously reported for this variant clone at pH 7.2 (2200 [2]) indicating that the same receptor was studied under all conditions.

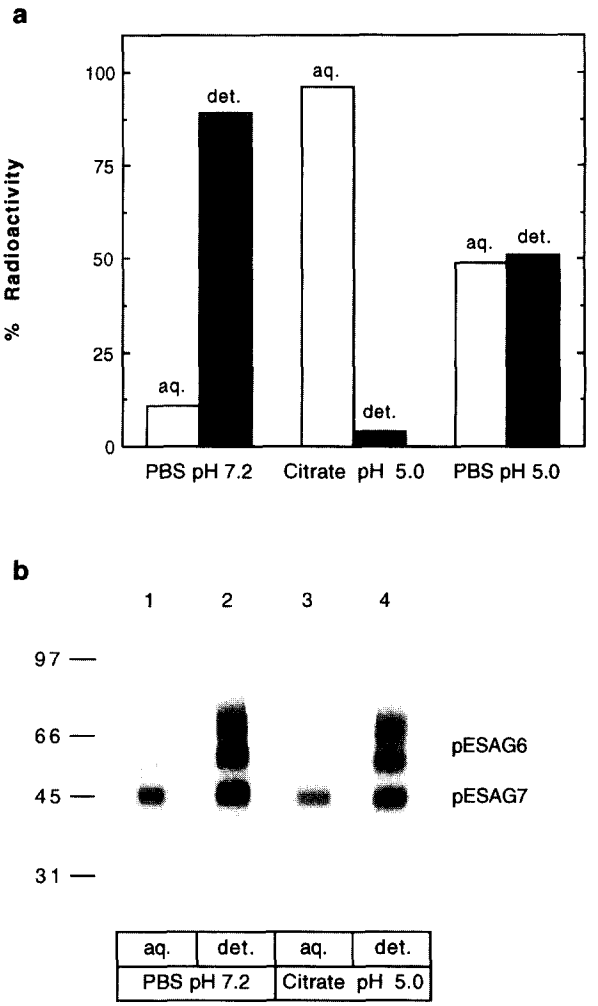

Fig. 1. Partition of Tf (a) and trypanosome TfR (b) into aqueous and detergent-rich phases after Triton X-114 phase separation. Trypanosome TfR, purified in the presence of PCMBS and lauryl sulfobetaine in order to inhibit the endogenous phospholipase C, was saturated with holo-^{3}H-Tf and the resulting ternary complex subjected to a Triton X-114 phase separation in the buffer as indicated. (a) Percentage of radioactivity associated with the aqueous (aq.) and detergent-rich (det.) phases. Background values determined by Triton X-114 phase separation with ^{3}H-Tf in the absence of TfR (see Section 2) have been subtracted. (b) Detection of trypanosome TfR (pESAG6 and pESAG7) in the aqueous (aq.) and detergent-rich (det.) phases. Shown is an immunoblot probed with anti-trypanosome TfR antibodies [2]. The molecular size of standard proteins in kDa is indicated.

4. Discussion

Previously it was shown by elution experiments using immobilised Tf that ternary complexes of trypanosome TfR with either holo-Tf or apo-Tf are stable at pH 5.0 [2]. In that approach, however, Tf was in large excess which may have shifted the equilibrium of complex formation in favour of the ternary complex. By using Triton X-114 phase separation we now demonstrate that the trypanosome TfR exhibits a low affinity for apo-Tf at pH 5.0. Furthermore, Scatchard analysis of binding data obtained with radiolabeled Tf and membranes of *T. brucei* at this pH revealed a high K_d value (1.1 μM) confirming the low affinity of the trypanosome TfR to its iron-free ligand at pH 5.0. In contrast, the mammalian TfR shows a high affinity for apo-Tf at this pH (K_d = 13–21 nM

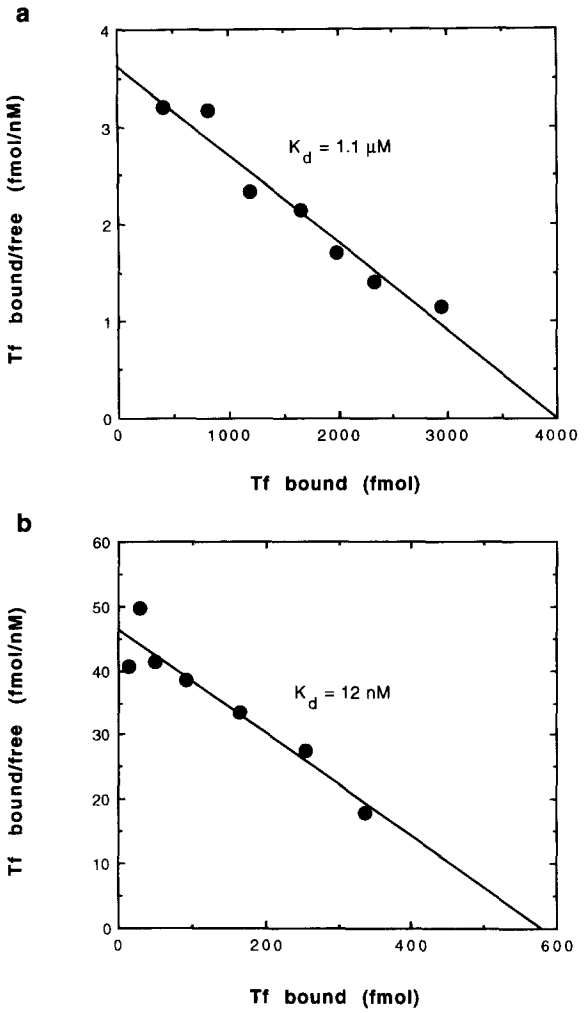

Fig. 2. Scatchard analysis of binding of apo-^{3}H-Tf (a) and holo-^{3}H-Tf (b) to membranes of *T. brucei* clone MITat 1.4 at pH 5.0. Binding assays were performed with (a) 10^9 cells in citrate buffer and (b) 1.5×10^8 cells in PBS as described in Section 2.

6,8]). For holo-Tf at pH 5.0, the K_d value for trypanosome TfR (12 nM) is similar to the value found for holo-Tf and mammalian TfR (13 nM [8]).

The different affinities of the trypanosome and mammalian TfR to apo-Tf at pH 5.0 explains the different fate of Tf in both types of cells. In mammalian cells, the receptor-ligand complex is delivered to endosomes where the low pH triggers the release of iron from Tf. Apo-Tf remains tightly bound to its receptor and is recycled to the cell surface to mediate further cycles of iron uptake [6,8]. Presumably also in trypanosomes the receptor-ligand complex is delivered to an endosomal system like other internalised macromolecules [15–17]. The acidic environment of this compartment certainly leads to liberation of iron from Tf and, because of the low affinity of the resulting apo-Tf for its receptor (see Figs. 1 and 2a), subsequently to dissociation of the complex. While Tf is transported to lysosomes where it is proteolytically digested [2,9], the unoccupied receptor is recycled as internalisation and degradation of ligand exceeds the total receptor content [2]. This mechanism, which resembles the uptake of low density lipoproteins and asialoglycoproteins by their specific receptors in mammalian cells [18,19], is more reasonable than the previous suggestion that metabolically stable trypanosome TfR accompanies its ligand to lysosomes [2].

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References

[1] Steverding, D., Stierhof, Y.-D., Chaudhri, M., Ligtenberg, M., Schell, D., Beck-Sickinger, A.G. and Overath, P. (1994) Eur. J. Cell Biol. 64, 78–87.
[2] Steverding, D., Stierhof, Y.-D., Fuchs, H., Tauber, R. and Overath, P. (1995) J. Cell Biol. 131, 1173–1182.
[3] Chaudhri, M., Steverding, D., Kittelberger, D., Tjia, S. and Overath, P. (1994) Proc. Natl. Acad. Sci. USA 91, 6443–6447.
[4] Ligtenberg, M.J.L., Bitter, W., Kieft, R., Steverding, D., Janssen, H., Calafat, J. and Borst, P. (1994) EMBO J. 13, 2565–2573.
[5] Salmon, D., Geuskens, M., Hanocq, F., Hanocq-Quertier, J., Nolan, D., Ruben, L. and Pays, E. (1994) Cell 78, 75–86.
[6] Klausner, R.D., Ashwell, G., van Renswoude, J., Harford, J.B. and Bridges, K.R. (1983) Proc. Natl. Acad. Sci. USA 80, 2263–2266.
[7] Octave, J.-N., Schneider, Y.-J., Crichton, R.R. and Trouet, A. (1981) Eur. J. Biochem. 115, 611–618.
[8] Dautry-Varsat, A., Ciechanover, A. and Lodish, H.F. (1983) Proc. Natl. Acad. Sci. USA 80, 2258–2262.
[9] Grab, D.J., Wells, C.W., Shaw, M.K., Webster, P. and Russo, D.C.W. (1992) Eur. J. Cell Biol. 59, 398–404.
[10] Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607.
[11] Cross, G.A.M. (1975) Parasitology 71, 393–417.
[12] Lanham, S.M. and Godfrey, D.G. (1970) Exp. Parasitol. 28, 521–534.
[13] Van Driel, I.R., Stearne, P.A., Grego, B., Simpson, R.J. and Goding, J.W. (1984) J. Immunol. 133, 3220–3224.
[14] Anderson, G.J., Mackerras, A., Powell, L.W. and Halliday, J.W. (1986) Biochim. Biophys. Acta 884, 225–233.
[15] Langreth, S.G. and Balber, A.E. (1975) J. Protozool. 22, 40–53.
[16] Webster, P. (1989) Eur. J. Cell Biol. 49, 295–302.
[17] Webster, P. and Fish, W.R. (1989) Eur. J. Cell Biol. 49, 303–310.
[18] Goldstein, J.L. and Brown, M.S. (1977) Annu. Rev. Biochem. 46, 897–930.
[19] Bridges, K., Harford, J., Ashwell, G. and Klausner, R.D. (1982) Proc. Natl. Acad. Sci. USA 79, 350–354.